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Date of Application :	June 16, 2003
Application Number :	Patent Application No. 2003-170326 [JP2003-170326]
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July 22, 2004

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Commissioner, Patent Office

[Title]	Application for Patent
[Reference Number]	A31369A
[Filing Date]	June 16, 2003
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[Number of register of payment]	170347
[Amount]	21,000
[List of Attached Documents]	
[Document's Name]	Specification 1
[Document's Name]	Drawings 1
[Document's Name]	Abstract 1
[Number of Comprehensive Power of Attorney]	0205404
[Proof]	Required

[Name of Document] SPECIFICATION

[Title of Invention] FLUORESCENT PROTEIN

[Claims] A fluorescent protein derived from Acropora sp., which has the following properties:

[1] the excitation maximum wavelength is 472 nm;

[2] the fluorescence maximum wavelength is 496 nm;

[3] the molar absorption coefficient at 472 nm is 27,250;

[4] the quantum yield is 0.90; and

[5] the pH sensitivity of light absorption property is pKa of approximately 6.6.

[Claim 2] A fluorescent protein having either the following amino acid sequences:

(a) an amino acid sequence shown in SEQ ID NO: 1; or

(b) an amino acid sequence, which comprises a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, and which has a fluorescence.

[Claim 3] DNA encoding the protein according to claim 1 or 2.

[Claim 4] DNA of either the following:

(a) DNA encoding an amino acid sequence shown in SEQ ID NO: 1; or

(b) DNA which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, and which encodes a fluorescent protein.

[Claim 5] DNA having either the following:

(a) a nucleotide sequence shown in SEQ ID NO: 2; or

(b) a nucleotide sequence which comprises a deletion, substitution, and/or addition of one or several nucleotides with respect to the nucleotide sequence shown in SEQ ID NO: 2, and which encodes a fluorescent protein.

[Claim 6] A recombinant vector having the DNA according to claim 4 or 5.

[Claim 7] A transformant having the DNA according to claim 4 or 5 or the recombinant

vector according to claim 6.

[Claim 8] A fusion fluorescent protein, which consists of the fluorescent protein according to claim 1 or 2, and another protein.

[Claim 9] The fusion fluorescent protein according to claim 8, wherein another protein is a protein that localizes in a cell.

[Claim 10] The fusion fluorescent protein according to claim 8 or 9, wherein another protein is a protein specific to a cell organella.

[Claim 11] A method for analyzing the localization or dynamics of a protein in a cell, which is characterized in that the fusion fluorescent protein according to claim 1 or 2 is allowed to express in the cell.

[Claim 12] A fluorescent reagent kit, which comprises: the fluorescent protein of claim 1 or 2; the DNA of claims 3 to 5; the recombinant vector of claim 6; the transformant of claim 7; or the fusion fluorescent protein of any of claims 8 to 10.

[Detailed Dscription of the Invention]

[0001]

[TECHNICAL FIELD OF THE INVENTION]

The present invention relates to a novel fluorescent protein. More specifically, the present invention relates to a novel fluorescent protein derived from Acropora sp., and the use thereof.

[0002]

[PRIOR ART]

Green fluorescent protein (GFP) derived from Aequorea victoria, a jellyfish, has many purposes in biological systems. Recently, various GFP mutants have been produced based on the random mutagenesis and semi-rational mutagenesis, wherein a color is changed, a folding property is improved, luminance is enhanced, or pH sensitivity is modified. Fluorescent proteins such as GFP are fused with other proteins by gene recombinant technique, and monitoring of the expression and transportation of

the fusion proteins is carried out.

[0003]

One of the most commonly used types of GFP mutant is Yellow fluorescent protein (YFP). Among Aequorea-derived GFP mutants, YFP exhibits the fluorescence with the longest wavelength. The values ε and Φ of the majority of YEPs are 60,000 to 100,000 $M^{-1}cm^{-1}$ and 0.6 to 0.8, respectively (Tsien, R. Y. (1998). Ann. Rev. Biochem. 67, 509-544). These values are comparable to those of the general fluorescent group (fluorescein, rhodamine, etc.). Accordingly, improvement of the absolute luminance of YFP is nearly approaching its limit.

[0004]

In addition, cyan fluorescent protein (CFP) is another example of the GFP mutant. Of this type of protein, ECFP (enhanced cyan fluorescent protein) has been known. Moreover, red fluorescent protein (RFP) has been isolated from sea anemone (Discoma sp.). Of this type of protein, DasRed has been known. Thus, 4 types of fluorescent proteins, that are, green fluorescent protein, yellow fluorescent protein, cyan fluorescent protein, and red fluorescent protein, have successively been developed. The range of the spectrum has significantly been expanded.

[0005]

In addition, some cnidarians emit a fluorescence. The cloning of fluorescent protein genes derived from such cnidarians has been attempted. However, in order to expand the repertoire of fluorescent and biochemical properties, it is necessary to clone more genes.

[0006]

[Non-patent Document 1] Tsien, R. Y. (1998). Ann. Rev. Biochem. 67, 509-544

[0007]

[OBJECT TO BE SOLVED BY THE INVENTION]

It is an object of the present invention to provide a novel fluorescent protein

derived from Acropora sp. Especially, it is an object of the present invention to provide a fluorescent protein having a spectrum property which is intermediate between conventional GFP and CFP.

[0008]

[MEANS FOR SOLVING THE OBJECT]

The present inventors have conducted intensive studies directed towards achieving the aforementioned objects. The inventors have designed preferred primers based on information regarding the amino acid sequences of known fluorescent proteins. Thereafter, using the aforementioned primers, the inventors have succeeded in amplifying gene encoding novel fluorescent protein obtained from cDNA library derived from Acropora sp., and in cloning them. Moreover, the present inventors have analyzed the fluorescence properties and pH sensitivity of the obtained fluorescent protein derived from Acropora sp. The present invention has been completed based on these findings.

[0009]

Thus, the present invention provides a fluorescent protein derived from Acropora sp., which has the following properties:

[1] the excitation maximum wavelength is 472 nm;

[2] the fluorescence maximum wavelength is 496 nm;

[3] the molar absorption coefficient at 472 nm is 27,250;

[4] the quantum yield is 0.90; and

[5] the pH sensitivity of light absorption property is pKa of approximately 6.6.

[0010]

Another aspect of the present invention provides a fluorescent protein having either the following:

(a) an amino acid sequence shown in SEQ ID NO: 1; or

(b) an amino acid sequence, which comprises a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID

NO: 1, and which has a fluorescence.

[0011]

Further another aspect of the present invention provides a DNA encoding the protein according of the present invention.

Further another aspect of the present invention provides DNA of either the following:

(a) DNA encoding an amino acid sequence shown in SEQ ID NO: 1; or

(b) DNA which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, and which encodes a fluorescent protein.

[0012]

Further another aspect of the present invention provides DNA having either the following nucleotide sequence:

(a) a nucleotide sequence shown in SEQ ID NO: 2; or

(b) a nucleotide sequence which comprises a deletion, substitution, and/or addition of one or several nucleotides with respect to the nucleotide sequence shown in SEQ ID NO: 2, and which encodes a fluorescent protein.

[0013]

Further another aspect of the present invention provides a recombinant vector having the DNA of the present invention.

Further another aspect of the present invention provides a transformant having the DNA or recombinant vector of the present invention.

[0014]

Further another aspect of the present invention provides a fusion fluorescent protein, which consists of the fluorescent protein of the present invention and another protein.

Another protein is preferably a protein that localizes in a cell, and more

preferably a protein specific to a cell organella.

[0015]

Further another aspect of the present invention provides a method for analyzing the localization or dynamics of a protein in a cell, which is characterized in that the fusion fluorescent protein of the present invention is allowed to express in the cell.

[0016]

Further another aspect of the present invention provides a fluorescent reagent kit, which comprises the fluorescent protein, DNA, recombinant vector, transformant or fusion fluorescent protein of the present invention.

[0017]

[EMBODIMENT FOR CARRYING OUT THE INVENTION]

The embodiments of the present invention will be described in detail below.

(1) Fluorescent proteins of the present invention

The fluorescent protein of the present invention is derived from Acropora sp., and it has the following properties:

[1] the excitation maximum wavelength is 472 nm;

[2] the fluorescence maximum wavelength is 496 nm;

[3] the molar absorption coefficient at 472 nm is 27,250;

[4] the quantum yield is 0.90; and

[5] the pH sensitivity of light absorption property is pKa of approximately 6.6.

[0018]

Acropora sp. is one kind of coral, which belongs to Cnidaria, Anthozoa, Hexacorallia, Seleractinia, Artrocoexniidae. This coral often forms a ramiform or tabular colony.

[0019]

As described in examples below, the fluorescent protein of the present invention has an excitation maximum wavelength of 472 nm and a fluorescence maximum

wavelength of 496 nm. In addition, its molar absorption coefficient at 472 nm is 27,250, and its quantum yield is 0.90. The molar absorption coefficient represents the amount of photon absorbed per mole of a fluorescent molecule. The quantum yield is a numerical value representing the degree of the absorbed photon that can be emitted as a fluorescence.

[0020]

A specific example of the fluorescent protein of the present invention is a fluorescent protein having either the following amino acid sequence:

(a) the amino acid sequence shown in SEQ ID NO: 1; or

(b) an amino acid sequence, which comprises a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, and which has fluorescence.

[0021]

The scope of "one or several" in the phrase "an amino acid sequence comprising a deletion, substitution and/or addition of one or several amino acids" used herein is not particularly limited. For example, it means 1 to 20, preferably 1 to 10, more preferably 1 to 7, further preferably 1 to 5, and particularly preferably 1 to 3.

[0022]

The term "has a fluorescence" covers all of the cases where any fluorescence is given. Various properties such as fluorescence intensity, excitation wavelength, fluorescence wavelength or pH sensitivity, may be changed or may remain unchanged, as compared with the case of the protein having an amino acid sequence shown in SEQ ID NO: 1.

[0023]

The method of obtaining the fluorescent protein of the present invention is not particularly limited. The proteins may be either a protein synthesized by chemosynthesis, or recombinant protein produced by a gene recombination technique.

Where a recombinant protein is produced, it is necessary to obtain DNA encoding the protein. Appropriate primers are designed by using information regarding the amino acid sequence shown in SEQ ID NO: 1 of the sequence listing of the present specification and the nucleotide sequence shown in SEQ ID NO: 2 thereof. Using these primers, PCR is carried out by using cDNA library derived from Acropora sp. as a template, so that DNA encoding the fluorescent protein of the present invention can be obtained. Where a partial fragment of DNA encoding the fluorescent protein of the present invention is obtained by the above-described PCR, the produced DNA fragments are ligated to one another by a gene recombination technique, so that DNA encoding the desired fluorescent protein can be obtained. The fluorescent protein of the present invention can be produced by introducing this DNA into an appropriate expression system. Expression in an expression system will be described later in the present specification.

[0024]

(2) DNA of the present invention

The present invention provides genes encoding the fluorescent proteins of the present invention.

A specific example of DNA encoding the fluorescent proteins of the present invention is either the following DNA:

(a) DNA encoding an amino acid sequence shown in SEQ ID NO: 1; or

(b) DNA, which encodes an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, and which encodes a fluorescent protein.

Another specific example of DNA encoding the florescent proteins of the present invention is either the following DNA:

(a) DNA having the nucleotide sequence shown in SEQ ID NO: 2; or

(b) DNA, which has a nucleotide sequence comprising a deletion, substitution, and/or

addition of one or several nucleotides with respect to the nucleotide sequence shown in SEQ ID NO: 2, and which encodes a fluorescent protein.

[0025]

The DNA of the present invention can be synthesized by, for example, the phosphoamidite method, or it can also be produced by polymerase chain reaction (PCR) using specific primers. The DNA of the present invention or its fragment is produced by the method described above in the specification.

[0026]

A method of introducing a desired mutation into a certain nucleic acid sequence is known to a person skilled in the art. For example, known techniques such as a site-directed mutagenesis, PCR using degenerated oligonucleotides, or the exposure of cells containing nucleic acid to mutagens or radioactive rays, are appropriately used, so as to construct DNA having a mutation. Such known techniques are described in, for example, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY., 1989; and Current Protocols in Molecular Biology, Supplements 1 to 38, John Wiley & Sons (1987-1997).

[0027]

(3) Recombinant vector of the present invention

The DNA of the present invention can be inserted into a suitable vector and used. The type of a vector used in the present invention is not particularly limited. For example, it may be either a vector that can autonomously replicate (e.g., a plasmid, etc.), or vector that is incorporated into the genomes of host cells when it is introduced into the host cells and is then replicated together with the chromosome into which it is incorporated.

[0028]

The vector used in the present invention is preferably an expression vector. In an expression vector, elements necessary for transcription (e.g., a promoter, etc.) are

functionally ligated to the DNA of the present invention. The promoter is a DNA sequence which shows a transcriptional activity in host cells, and it is appropriately selected depending on the type of host cells.

[0029]

Examples of a promoter which can operate in bacterial cells may include a *Bacillus stearothermophilus* maltogenic amylase gene promoter, a *Bacillus licheniformis* alpha-amylase gene promoter, a *Bacillus amyloliquefaciens* BAN amylase gene promoter, a *Bacillus subtilis* alkaline protease gene promoter, a *Bacillus pumilus* xylosidase gene promoter, P_R and P_L promoters of phage rhamda, and lac, trp and tac promoters of *Escherichia coli*.

[0030]

Examples of a promoter which can operate in mammalian cells may include an SV40 promoter, an MT-1 (metallothionein gene) promoter, and an adenovirus-2 major late promoter. Examples of a promoter which can operate in insect cells may include a polyhedrin promoter, a P10 promoter, an *Autographa californica* polyhedrosis basic protein promoter, a baculovirus immediate-early gene 1 promoter, and a baculovirus 39K delayed-early gene promoter. Examples of a promoter which can be operate in yeast host cells may include promoters derived from yeast glycolytic genes, an alcohol dehydrogenase gene promoter, a TPI1 promoter, and an ADH2-4c promoter.

Examples of a promoter which can operate in filamentous cells may include an ADH3 promoter and a tpiA promoter.

[0031]

In addition, an appropriate terminator such as a human growth hormone terminator, or a TPI1 terminator or ADH3 terminator for fungal cells, may be functionally bound to the DNA of the present invention, as necessary. The recombinant vector of the present invention may further have elements such as a polyadenylation signal (e.g., one derived from SV40 or the adenovirus 5E1b region), a transcription

enhancer sequence (e.g., an SV40 enhancer), or a translation enhancer sequence (e.g., one encoding the adenovirus VA RNA).

The recombinant vector of the present invention may further comprise a DNA sequence which enables the replication of the recombinant vector in host cells. SV40 replication origin is an example of such a sequence (when the host cells are mammalian cells).

[0032]

The recombinant vector of the present invention may further comprise a selective marker. Examples of such a selective marker may include genes, complements of which are absent from host cells, such as a dihydrofolate reductase (DHFR) gene or a *Shizosaccharomyces pombe* TPI gene, and drug resistant genes such as ampicillin, kanamycin, tetracycline, chloramphenicol, neomycin or hygromycin-resistant genes.

A method for ligating the DNA of the present invention, a promoter and, as desired, a terminator and/or a secretory signal sequence to one another and inserting these items into a suitable vector is known to a person skilled in the art.

[0033]

(4) Transformant of the present invention

A transformant can be produced by introducing the DNA or recombinant vector of the present invention into a suitable host.

Any cell can be used as a host cell into which the DNA or recombinant vector of the present invention is introduced, as long as the DNA construct of the present invention can be expressed therein. Examples of such a cell may include bacteria, yeasts, fungal cells, and higher eukaryotic cells.

[0034]

Examples of bacteria may include Gram-positive bacteria such as Bacillus or Streptomyces, and Gram-negative bacteria such as *Escherichia coli*. These bacteria

may be transformed by the protoplast method or other known methods, using competent cells.

Examples of mammalian cells may include HEK 293 cells, HeLa cells, COS cells, BHK cells, CHL cells, and CHO cells. A method of transforming mammalian cells and expressing the introduced DNA sequence in the cells is also known. Examples of such a method may include the electroporation, the calcium phosphate method, and the lipofection method.

[0035]

Examples of yeast cells may include those belonging to Saccharomyces or Shizosaccharomyces. Examples of such cells may include *Saccharomyces cerevisiae* and *Saccharomyces kluyveri*. Examples of a method of introducing a recombinant vector into yeast host cells may include the electroporation, the spheroplast method, and the lithium acetate method.

[0036]

Examples of other fungal cells may include those belonging to *Filamentous fungi* such as Aspergillus, Neurospora, Fusarium or Trichoderma. Where *Filamentous fungi* are used as host cells, transformation can be carried out by incorporating DNA constructs into host chromosomes, so as to obtain recombinant host cells. Incorporation of DNA constructs into the host chromosomes is carried out by known methods, and such known methods may include homologous recombination and heterologous recombination.

[0037]

Where insect cells are used as host cells, both a vector into which a recombinant gene is introduced and a baculovirus are co-introduced into insect cells, and a recombinant virus is obtained in the culture supernatant of the insect cells. Thereafter, insect cells are infected with the recombinant virus, so as to allow the cells to express proteins (described in, for example, Baculovirus Expression Vectors, A Laboratory

Manual; and Current Protocols in Molecular Biology, Bio/Technology, 6, 47 (1988)).

[0038]

The *Autographa californica* nuclear polyhedrosis virus, which is a virus infecting to insects belonging to *Barathra brassicae*, can be used as baculovirus.

Examples of insect cells used herein may include Sf9 and Sf21, which are *Spodoptera frugiperda* ovarian cells [Baculovirus Expression Vectors, A Laboratory Manual, W. H. Freeman & Company, New York, (1992)], and HiFive (manufactured by Invitrogen), which are *Trichoplusia ni* ovarian cells.

Examples of the method of co-introducing both a vector into which a recombinant gene has been introduced and the above baculovirus into insect cells to prepare a recombinant virus may include the calcium phosphate method and the lipofection method.

[0039]

The above transformant is cultured in an appropriate nutritive medium under conditions enabling the introduced DNA construct to be expressed. In order to isolate and purify the protein of the present invention from the culture product of the transformant, common methods of isolating and purifying proteins may be used.

For example, where the protein of the present invention is expressed in a state dissolved in cells, after completion of the culture, cells are recovered by centrifugal separation, and the recovered cells are suspended in a water type buffer. Thereafter, the cells are disintegrated using an ultrasonic disintegrator or the like, so as to obtain a cell-free extract. A supernatant is obtained by centrifuging the cell-free extract, and then, a purified sample can be obtained from the supernatant by applying, singly or in combination, the following ordinary protein isolation and purification methods: the solvent extraction, the salting-out method using ammonium sulfate or the like, the desalting method, the precipitation method using an organic solvent, the anion exchange chromatography using resins such as diethylaminoethyl (DEAE) sepharose, the cation

exchange chromatography using resins such as S-Sepharose FF (manufactured by Pharmacia), the hydrophobic chromatography using resins such as butyl sepharose or phenyl sepharose, the gel filtration method using a molecular sieve, the affinity chromatography, the chromatofocusing method, and the electrophoresis such as isoelectric focusing.

[0040]

(5) Use of the fluorescent protein of the present invention and a fusion fluorescent protein comprising the same

The fluorescent protein of the present invention can be fused with another protein, so as to construct a fusion fluorescent protein.

A method of obtaining the fusion fluorescent protein of the present invention is not particularly limited. It may be either a protein synthesized by chemosynthesis, or recombinant protein produced by a gene recombination technique.

Where a recombinant protein is produced, it is necessary to obtain DNA encoding the protein. Appropriate primers are designed using the information regarding the amino acid sequence shown in SEQ ID NO: 1 of the sequence listing of the present specification and the nucleotide sequence shown in SEQ ID NO: 2 thereof. Using these primers, PCR is carried out using a DNA fragment containing the gene of the fluorescent protein of the present invention as a template, so as to produce DNA fragments necessary for construction of the DNA encoding the fluorescent protein of the present invention. Moreover, DNA fragment encoding a protein to be fused is also obtained in the same above manner.

[0041]

Subsequently, the thus obtained DNA fragments are ligated to one another by a gene recombination technique, so that DNA encoding the desired fusion fluorescent protein can be obtained. This DNA is then introduced into an appropriate expression system, so that the fusion fluorescent protein of the present invention can be produced.

[0042]

The fluorescent protein of the present invention has an extremely high utility value as a marker. This is to say, the fluorescent protein of the present invention is purified as a fusion protein with an amino acid sequence to be tested, and the fusion protein is introduced into cells by methods such as the microinjection. By observing the distribution of the fusion protein over time, targeting activity of the amino acid sequence to be tested can be detected in the cells.

[0043]

The type of another protein (an amino acid sequence to be tested) with which the fluorescent protein of the present invention is fused is not particularly limited. Preferred examples may include proteins localizing in cells, proteins specific for intracellular organelles, and targeting signals (e.g., a nuclear transport signal, a mitochondrial presequence, etc.). In addition, the fluorescent protein of the present invention can be expressed in cells and used, as well as being introduced into cells by the microinjection or the like. In this case, a vector into which the DNA encoding the fluorescent protein of the present invention is inserted in such a way that it can be expressed, is introduced into host cells.

[0044]

Moreover, the fluorescent protein of the present invention can also be used as a reporter protein to determine promoter activity. This is to say, a vector is constructed such that DNA encoding the fluorescent protein of the present invention is located downstream of a promoter to be tested, and the vector is then introduced into host cells. By detecting the fluorescence of the fluorescent protein of the present invention which is emitted from the cells, the activity of the promoter to be tested can be determined. The type of a promoter to be tested is not particularly limited, as long as it operates in host cells.

[0045]

A vector used to detect the targeting activity of the above amino acid sequence to be tested or to determine promoter activity is not particularly limited. Examples of a vector preferably used for animal cells may include pNEO (P. Southern, and P. Berg (1982) J. Mol. Appl. Genet. 1: 327), pCAGGS (H. Niwa, K. Yamamura, and J. Miyazaki, Gene 108, 193-200 (1991)), pRc/CMV (manufactured by Invitrogen), and pCDM8 (manufactured by Invitrogen). Examples of a vector preferably used for yeasts may include pRS303, pRS304, pRS305, pRS306, pRS313, pRS314, pRS315, pRS316 (R. S. Sikorski and P. Hieter (1989) Genetics 122: 19-27), pRS423, pRS424, pRS425, pRS426 (T. W. Christianson, R. S. Sikorski, M. Dante, J. H. Shero, and P. Hieter (1992) Gene 110: 119-122).

[0046]

In addition, the type of cells used herein is also not particularly limited. Various types of animal cells such as L cells, BalbC-3T3 cells, NIH3T3 cells, CHO (Chinese hamster ovary) cells, HeLa cells or NRK (normal rat kidney) cells, yeast cells such as *Saccharomyces cerevisiae*, *Escherichia coli* cells, or the like can be used. Vector can be introduced into host cells by common methods such as the calcium phosphate method or the electroporation.

[0047]

The above obtained fusion fluorescent protein of the present invention wherein the fluorescent protein of the present invention is fused with another protein (referred to as a protein X) is allowed to be expressed in cells. By monitoring a fluorescence emitted, it becomes possible to analyze the localization or dynamics of the protein X in cells. That is, cells transformed or transfected with DNA encoding the fusion fluorescent protein of the present invention are observed with a fluorescence microscope, so that the localization and dynamics of the protein X in the cells can be visualized and thus analyzed.

[0048]

For example, by using a protein specific for an intracellular organella as a protein X, the distribution and movement of a nucleus, a mitochondria, an endoplasmic reticulum, a Golgi body, a secretory vesicle, a peroxisome, etc., can be observed.

Moreover, for example, axis cylinders or dendrites of the nerve cells show an extremely complicated change in strikes in an individual who is under development. Accordingly, fluorescent labeling of these sites enables a dynamic analysis.

[0049]

The fluorescence of the fluorescent protein of the present invention can be detected with a viable cell. Such detection can be carried out using, for example, a fluorescence microscope (Axiophoto Filter Set 09 manufactured by Carl Zeiss) or an image analyzer (Digital Image Analyzer manufactured by ATTO).

The type of a microscope can be appropriately selected depending on purposes. Where frequent observation such as pursuit of a change over time is carried out, an ordinary incident-light fluorescence microscope is preferable. Where observation is carried out while resolution is emphasized, for example, in the case of searching localization in cells specifically, a confocal laser scanning microscope is preferable. In terms of maintenance of the physiological state of cells and prevention from contamination, an inverted microscope is preferable as a microscope system. When an erecting microscope with a high-powered lens is used, a water immersion lens can be used.

[0050]

A filter set can be appropriately selected depending on the fluorescence wavelength of a fluorescent protein. In the case of the fluorescent proteins of the present invention, a filter having an excitation light between approximately 460 and 480 nm and a fluorescence between approximately 480 and 510 nm is preferably used.

[0051]

When viable cells are observed over time using a fluorescence microscope, a

high sensitive cooled CCD camera is used, since photography is carried out in a short time. In the case of the cooled CCD camera, CCD is cooled to decrease thermal noise, so that a weak fluorescence image can be clearly photographed by exposure in a short time.

[0052]

(6) Kit of the present invention

The present invention provides a kit for analyzing the localization of intracellular components and/or analyzing physiologically active substances, which is characterized in that it comprises at least one selected from the fluorescent protein, the fusion fluorescent protein, the DNA, the recombinant vector, or the transformant, which are described in the present specification. The kit of the present invention can be produced from commonly used materials that are known per se, by using common methods.

Reagents such as thefluorescent protein or the DNA are dissolved in an appropriate solvent, so that the reagents can be prepared in a form suitable for conservation. Water, ethanol, various types of buffer solution, etc. can be used as such a solvent.

The present invention will be further described in the following examples. However, the present invention is not limited by these examples.

[0053]

[EXAMPLES]

Example 1: Isolation of novel fluorescent protein gene (MICy) from coral

(1) Extraction of total RNA

A fluorescent protein gene was isolated from coral emitting a fluorescence. Acropora sp. was used as a material. Acropora sp. was crushed with a hammer, and 15 ml of "TRIzol" (GIBCO BRL) was then added to 5 g of the crushed Acropora sp. Thereafter, the obtained mixture was stirred and then centrifuged at 1,500 x g for 10

minutes. Thereafter, 3 ml of chloroform was added to the obtained supernatant, and the mixture was then stirred for 15 seconds. Thereafter, the mixture was left at rest for 3 minutes. The resultant was then centrifuged at 7,500 x g for 15 minutes. Thereafter, 7.5 ml of isopropanol was added to the obtained supernatant, and the mixture was then stirred for 15 seconds, followed by leaving the mixture at rest for 10 minutes. Thereafter, the resultant was centrifuged at 17,000 x g for 10 minutes. The obtained supernatant was discarded, and 6 ml of 70% ethanol was added to the residue, followed by centrifugation at 17,000 x g for 10 minutes. The obtained supernatant was discarded, and the precipitate was then dissolved in 200 μl of DEPC water. Total RNA dissolved in the DEPC water was 100 times diluted, and the values of O.D.260 and O.D.280 were then measured, so as to determine RNA concentration. As a result, 220 μg of total RNA was obtained.

[0054]

(2) Synthesis of first strand cDNA

cDNA (33 μl) was synthesized from 5 μg of the total RNA, using a kit for synthesizing first strand cDNA "Ready To Go" (Amersham Pharmacia).

[0055]

(3) Degenerated PCR

3 μl of the synthesized first strand cDNA (33 μl) was used as a template to carry out PCR. Primers were produced by making comparison among the amino acid sequences of known fluorescent proteins, extracting similar portions, and converting them to nucleotide sequences.

Primers used:

5'-GAAGGRTGYGTCAAYGGRCAY-3' (primer 1) (SEQ ID NO: 3)

5'-ACVGGDCCATYDGVAAGAAARTT-3' (primer 2) (SEQ ID NO: 4)

R represents A or G; Y represents C or T; V represents A, C, or G; D represents A, G, or T

[0056]

Composition of PCR reaction solution:

Template (first strand cDNA)	3 μl
X10 taq buffer	5 μl
2.5 mM dNTPs	4 μl
100 μM primer 1	1 μl
100 μM primer 2	1 μl
Milli-Q	35 μl
taq polymerase (5 U/μl)	1 μl

[0057]

PCR reaction conditions:

94°C x 1 min (PAD)

94°C x 30 sec (denaturation)

52°C x 30 sec (annealing of primers to template)

72°C x 1 min (primer elongation)

A cycle consisting of the aforementioned 3 steps was repeated 30 times. The annealing temperature was decreased by 0.3°C for every cycle. The aneeling temperature was 43°C at the time of the 30th cycle.

72°C x 7 min (final elongation)

4°C (maintenance)

[0058]

Using 1 μl of the amplified product obtained in the first PCR reaction as a template, PCR was carried out again under the same above conditions. A band with an estimated size of 350 bp was cut out via agarose gel electrophoresis, and it was then purified.

[0059]

(4) Subcloning and sequencing

The purified DNA fragment was ligated to a pT7-blue vector (Novagen).

Escherichia coli (TG1) was transformed therewith, and it was then subjected to blue white selection. Plasmid DNA was purified from white colonies of Escherichia coli, and the nucleotide sequence of the inserted DNA fragment was determined using a DNA sequencer. Thereafter, the obtained nucleotide sequence was compared with the nucleotide sequences of other fluorescent protein genes, so as to determine whether the nucleotide sequence of the DNA fragment was derived from a fluorescent protein. With regard to those that were determined to be a part of the fluorescent protein genes, the full-length genes were cloned by the 5'-RACE method and the 3'-RACE method.

[0060]

(5) 5'-RACE method

In order to determine the nucleotide sequence on the 5'-side of the DNA fragment obtained by the degenerated PCR, the 5'-RACE method was applied using 5'-RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (GIBCO BRL). 3 μg of the total RNA prepared in (1) above was used as a template. The following primers were used in the first amplification of dC-tailed cDNA:

5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3' (primer 3) (SEQ ID NO: 17); and

5'- TAGAAATGACCTTTCATATGACATTC -3' (primer 4) (SEQ ID NO: 27).

Herein, I represents inosine.

The following primers were used in the second amplification:

5'-GGCCACGCGTCGACTAGTAC-3' (primer 5) (SEQ ID NO: 19); and

5'-TCTGTTTCCATATTGAAAGGCTG-3' (primer 6) (SEQ ID NO: 28). PCR reaction conditions were applied in accordance with protocols attached to the kit.

[0061]

The amplified 500-bp band was cut out of the gel via agarose gel electrophoresis and then purified. The purified DNA fragment was ligated to a pT7-blue vector (Novagen). Escherichia coli (TG1) was transformed therewith, and it was then

subjected to blue white selection. Plasmid DNA was purified from white colonies of Escherichia coli, and the nucleotide sequence of the inserted DNA fragment was determined using a DNA sequencer.

[0062]

(6) 3'-RACE method

The nucleotide sequence on the 3'-side of the DNA fragment obtained by the degenerated PCR was obtained by PCR using a primer prepared based on the information obtained by determination of the nucleotide sequence in (4) above and an oligo dT primer. 3 μl of the first strand cDNA prepared in (2) above was used as a template. The prepared primer was

5'-ATGGTGTCTTATTCAAAGCAAGGCATCGCACA-3' (primer 7) (SEQ ID NO: 29).

Composition of PCR reaction solution:

Template (first strand cDNA)	3 μl
X10 taq buffer	5 μl
2.5 mM dNTPs	4 μl
20 μM primer 7	1 μl
10 μM oligo dT primer	1 μl
Milli-Q	35 μl
Taq polymerase (5 U/μl)	1 μl

[0063]

PCR reaction conditions:

94°C x 1 minute (PAD)

94°C x 30 seconds (denaturation)

55°C x 30 seconds (annealing of primers to template)

72°C x 1 minute (primer elongation)

A cycle consisting of the aforementioned 3 steps was repeated 30 times.

72°C x 7 minutes (final elongation)

4°C (maintenance)

[0064]

The amplified band with a length of 900 bp was cut out of the gel via agarose gel electrophoresis and then purified. The purified DNA fragment was ligated to a pT7-blue vector (Novagen). Escherichia coli (TG1) was transformed therewith, and it was then subjected to blue white selection. Plasmid DNA was purified from white colonies of Escherichia coli, and the nucleotide sequence of the inserted DNA fragment was determined using a DNA sequencer.

The obtained full-length nucleotide sequence is shown in SEQ ID NO: 2 of the sequence listing, and the obtained full-length amino acid sequence is shown in SEQ ID NO: 1 thereof. This clone was named MICy.

[0065]

(7) Expression of protein in Escherichia coli

Using a primer produced with a portion corresponding to the N-terminus of the obtained full-length nucleotide sequence of the protein and an oligo dT primer, PCR was carried out employing the first strand cDNA prepared in (2) above as a template.

Primer used:

5'-CGGGATCCGACCATGGTGTCTTATTCAAAGCAAGGCATCGCACA-3' (primer 8) (SEQ ID NO: 10)

[0066]

Composition of PCR reaction solution:

Template (first strand cDNA)	3 μl
X10 pyrobest buffer	5 μl
2.5 mM dNTPs	4 μl
20 μM primer 8	1 μl
20 μM oligo dT primer	1 μl
Milli-Q	35 μl

Pyrobest polymerase (5 U/μl) 1 μl

[0067]

PCR reaction conditions:

94°C x 1 minute (PAD)

94°C x 30 seconds (denaturation)

55°C x 30 seconds (annealing of primers to template)

72°C x 1 minute (primer elongation)

A cycle consisting of the aforementioned 3 steps was repeated 30 times.

72°C x 7 minutes (final elongation)

4°C (maintenance)

[0068]

The amplified band with a length of 900 bp was cut out of the gel via agarose gel electrophoresis and then purified. The purified DNA fragment was subcloned into the BamHI-EcoRI site of a pRSET vector (Invitrogen), and it was then allowed to express in Escherichia coli (JM109-DE3). The expressed protein was constructed such that His-tag was attached to the N-terminus thereof, and thus it was purified with Ni-Agarose gel (QIAGEN). Purification was carried out in accordance with the attached protocols. Subsequently, the properties of the purified protein were analyzed.

[0069]

(8) Analysis of fluorescence properties

Using a solution comprising 20 μM fluorescent protein (MICy), 150 mM KCl, and 50 mM HEPES(pH 7.4), the absorption spectrum of the protein was measured (Figure 2). Thereafter, the molar absorption coefficient of the protein was calculated from the value of the peak (472 nm) of the spectrum. The fluorescent protein was diluted with the above buffer solution such that the absorption at 440 nm became 0.001. Its fluorescence spectrum by exciting at 440 nm and its excitation spectrum by a fluorescence at 540 nm were measured (Figure 1. Likewise, EGFP (CLONTECH) was

diluted such that the absorption at 440 nm became 0.001, and its fluorescence spectrum was measured. Setting the quantum yield of EGFP to 0.6, the quantum yield of the cloned fluorescent protein was obtained. The measurement results are shown in Table 1.

[0070]

[Table 1]

Table 1

	Excitation maximum	Fluorescence maximum	Molar absorption coefficient	Quantum yield	pH sensitivity	Number of amino acids
MICy	472 nm	496 nm	27,250 (472 nm)	0.90	pKa = 6.6	232

[0071]

(9) Measurement of pH sensitivity

The fluorescent protein was diluted with each of the following buffer solutions. The value of the absorption at 472 nm was determined, and thus the pH sensitivity thereof was measured. The pH levels of the buffer solutions are as follows.

pH 4 and 5: Acetate buffer

pH 6 and 11: Phosphate buffer

pH 7 and 8: HEPES buffer

pH 9 and 10: Glycine buffer

The measurement results are shown in Figure 3.

[0072]

[Effect of the Invention]

The present invention provides novel fluorescent proteins derived from Acropora sp.. The fluorescent protein of the present invention is a novel protein having a primary structure different from that of the conventional fluorescent proteins. The

fluorescent protein of the present invention has certain fluorescence properties, and thus it is useful for molecular biological analysis. In addition, a mutation is introduced into the fluorescent protein of the present invention, so as to generate new fluorescence properties.

[0073]

[SEQUENCE LISTING]

SEQUENCE LISTING

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<160> 10

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<213> Acropora sp.

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20 25 30

Val Gly Thr Gly Asn Pro Tyr Glu Gly Lys Gln Met Ser Glu Leu Val
35 40 45

Ile Ile Lys Ser Lys Gly Lys Pro Leu Pro Phe Ser Phe Asp Ile Leu
50 55 60

Ser Thr Ala Phe Gln Tyr Gly Asn Arg Cys Phe Thr Lys Tyr Pro Ala
65 70 75 80

Asp Met Pro Asp Tyr Phe Lys Gln Ala Phe Pro Asp Gly Met Ser Tyr

85 90 95

Glu Arg Ser Phe Leu Phe Glu Asp Gly Gly Val Ala Thr Ala Ser Trp

100 105 110

Ser Ile Arg Leu Glu Gly Asn Cys Phe Ile His Asn Ser Ile Tyr His

115 120 125

Gly Val Asn Phe Pro Ala Asp Gly Pro Val Met Lys Lys Gln Thr Ile

130 135 140

Gly Trp Asp Lys Ser Phe Glu Lys Met Ser Val Ala Lys Glu Val Leu

145 150 155 160

Arg Gly Asp Val Thr Gln Phe Leu Leu Leu Glu Gly Gly Gly Tyr Gln

165 170 175

Arg Cys Arg Phe His Ser Thr Tyr Lys Thr Glu Lys Pro Val Ala Met

180 185 190

Pro Pro Ser His Val Val Glu His Gln Ile Val Arg Thr Asp Leu Gly

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His Val Asn Pro Leu Lys Val Lys

225 230

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<213> Acropora sp.

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1 5 10 15

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Tyr Arg Met Glu Gly Ser Val Asn Gly His Glu Phe Thr Ile Glu Gly
            20                  25                  30

gta gga act gga aac cct tac gaa ggg aaa cag atg tcc gaa tta gtg 144
Val Gly Thr Gly Asn Pro Tyr Glu Gly Lys Gln Met Ser Glu Leu Val
        35                  40                  45

atc atc aag tct aag gga aaa ccc ctt cca ttc tcc ttt gac ata ctg 192
Ile Ile Lys Ser Lys Gly Lys Pro Leu Pro Phe Ser Phe Asp Ile Leu
    50                  55                  60

tca aca gcc ttt caa tat gga aac aga tgc ttc aca aag tac cct gca 240
Ser Thr Ala Phe Gln Tyr Gly Asn Arg Cys Phe Thr Lys Tyr Pro Ala
65                  70                  75                  80

gac atg cct gac tat ttc aag caa gca ttc cca gat gga atg tca tat 288
Asp Met Pro Asp Tyr Phe Lys Gln Ala Phe Pro Asp Gly Met Ser Tyr
                85                  90                  95

gaa agg tca ttt cta ttt gag gat gga gga gtt gct aca gcc agc tgg 336
Glu Arg Ser Phe Leu Phe Glu Asp Gly Gly Val Ala Thr Ala Ser Trp
            100                 105                 110

agc att cgt ctc gaa gga aat tgc ttc atc cac aat tcc atc tat cat 384
Ser Ile Arg Leu Glu Gly Asn Cys Phe Ile His Asn Ser Ile Tyr His
        115                 120                 125

ggc gta aac ttt ccc gct gat gga ccc gta atg aag aag cag aca att 432
Gly Val Asn Phe Pro Ala Asp Gly Pro Val Met Lys Lys Gln Thr Ile
    130                 135                 140

ggc tgg gat aag tcc ttc gaa aaa atg agt gtg gct aaa gag gtg cta 480
Gly Trp Asp Lys Ser Phe Glu Lys Met Ser Val Ala Lys Glu Val Leu
145                 150                 155                 160
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Arg Cys Arg Phe His Ser Thr Tyr Lys Thr Glu Lys Pro Val Ala Met
180 185 190
ccc ccg agt cat gtc gta gaa cat caa att gtg agg acc gac ctt ggc 624
Pro Pro Ser His Val Val Glu His Gln Ile Val Arg Thr Asp Leu Gly
195 200 205
caa act gca aaa ggc ttc aag gtc aag ctg gaa gaa cat gct gag gct 672
Gln Thr Ala Lys Gly Phe Lys Val Lys Leu Glu Glu His Ala Glu Ala
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[BRIEF DESCRIPTION OF THE DRAWINGS]

[Figure 1] Figure 1 shows the results of the measurements of the fluorescence

spectrum and excitation spectrum of the fluorescent protein (MICy) of the present invention derived from Acropora sp.

[Figure 2] Figure 2 shows the absorption spectrum of the fluorescent protein (MICy) of the present invention derived from Acropora sp.

[Figure 3] Figure 3 shows the pH sensitivity of the fluorescent protein (MICy) of the present invention derived from Acropora sp. The horizontal axis represents pH value, and the longitudinal axis represents absorbance.

[Name of Document] DRAWING

[Figure 1]

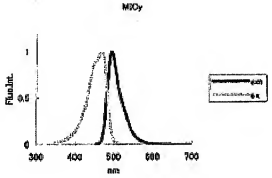


Figure 1 fluorescence spectrum and excitation spectrum

[Figure 2]

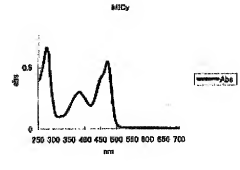
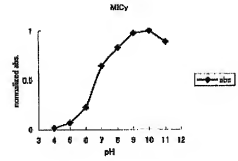


Figure 2 absorption spectrum

[Figure 3]



Fugure 3 pH sensitivity

[Name of Document] ABSTRACT

[Abstract]

[Object] To provide a novel fluorescent protein derived from Acropora sp.

[Means for Solution] A fluorescent protein derived from Acropora sp., which has the following properties:

[1] the excitation maximum wavelength is 472 nm;

[2] the fluorescence maximum wavelength is 496 nm;

[3] the molar absorption coefficient at 472 nm is 27,250;

[4] the quantum yield is 0.90; and

[5] the pH sensitivity of light absorption property is pKa of approximately 6.6.

[Seelected Drawing] None